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Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site

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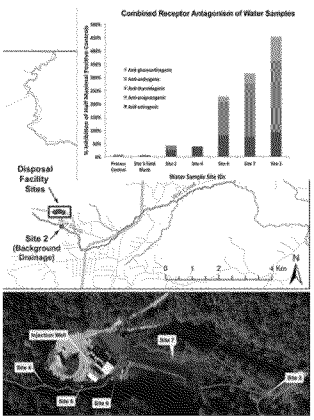
HIGHLIGHTS

- Oil and gas wastewater disposal may increase endocrine disrupting activity in water.
- Tested EDC activity in surface water near oil and gas wastewater injection site.
- Water downstream had significantly more EDC activity than reference water upstream.
- Downstream surface water antagonized five different nuclear hormone receptors.
- EDC activity downstream was above levels known to result in adverse health effects.

GRAPHICAL ABSTRACT



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abstract

Currently, N95% of end disposal of hydraulic fracturing wastewater from unconventional oil and gas operations in the US occurs via injection wells. Key data gaps exist in understanding the potential impact of underground injection on surface water quality and environmental health. The goal of this study was to assess endocrine disrupting activity in surface water at a West Virginia injection well disposal site. Water samples were collected from a background site in the area and upstream, on, and downstream of the disposal facility. Samples were solid-phase extracted, and extracts assessed for agonist and antagonist hormonal activities for five hormone receptors in mammalian and yeast reporter gene assays. Compared to reference water extracts upstream and distal to the disposal well, samples collected adjacent and downstream exhibited considerably higher antagonist activity for the estrogen, androgen, progesterone, glucocorticoid and thyroid hormone receptors. In contrast, low levels of agonist activity were measured in upstream/distal sites, and were inhibited or absent at downstream sites with significant antagonism. Concurrent analyses by partner laboratories (published separately) describe the

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Injection well
Wastewater disposal

analytical and geochemical profiling of the water; elevated conductivity as well as high sodium, chloride, strontium, and barium concentrations indicate impacts due to handling of unconventional oil and gas wastewater. Notably, antagonist activities in downstream samples were at equivalent authentic standard concentrations known to disrupt reproduction and/or development in aquatic animals. Given the widespread use of injection wells for end-disposal of hydraulic fracturing wastewater, these data raise concerns for human and animal health nearby.

1. Introduction

It has recently been demonstrated that chemicals used in and/or produced by unconventional oil and natural gas (UOG) operations include endocrine disrupting chemicals (EDCs) (Bolden et al., 2015; Kassotis et al., 2014; Webb et al., 2014). EDCs are exogenous chemicals or mixtures of chemicals that can interfere with any aspect of hormone action (Zoeller et al., 2012). As many as one thousand EDCs have been identified (TEDX, 2013), both synthetic and naturally occurring, that can directly interact with hormone receptors as agonists or antagonists (Tyler et al., 1998; Yang et al., 2006), or indirectly interact via modulating responses to endogenous hormones (Chen et al., 2007; Jansen et al., 2004), endogenous hormone levels (Chen et al., 2007; Hayes, 2002), or through other mechanisms (Diamanti-Kandarakis et al., 2009). EDCs can exhibit biological effects at very low environmental concentrations (Roepke et al., 2005), can exhibit non-monotonic response curves (quantitatively and qualitatively different outcomes at low versus high concentrations), and can alter development during critical windows and increase the risk of disease (Vandenberg et al., 2012; Welshons et al., 2003).

UOG extraction involves harvesting oil and natural gas reserves, including shale gas, coal bed methane, and shale oil, trapped in impermeable or low-permeability geologic layers. As such, extraction of these energy resources requires stimulation, routinely via processes such as hydraulic fracturing (high pressure injection of water, chemicals, and suspended solids), to fracture the target layer and release the trapped natural gas and/or oil (Waxman et al., 2011; Wiseman, 2008). While less than fifty chemicals are typically used for the hydraulic fracturing of a single well, there are approximately 1000 different chemicals used by industry across the US (US EPA, 2015; Waxman et al., 2011); of these, N100 are known or suspected EDCs (Colborn et al., 2011; Kassotis et al., 2014; Waxman et al., 2011). A small percentage of injected fluids are recovered as "flow back" over approximately the first two weeks, while "produced water" is then generated over the life of the producing well (Deutch et al., 2011; Engle et al., 2014). These wastewaters can be heavily laden with naturally occurring radioactive compounds, heavy metals, and other compounds from the shale layer (Akob et al., 2015; Rowan et al., 2015), as well as chemicals and compounds used and produced by fracturing operations, and are routinely injected into disposal wells, reused in future fracturing operations, and/or pumped into open evaporation pits for disposal (Deutch et al., 2011; Lee et al., 2011; Lester et al., 2015; Wiseman, 2008).

Economically feasible methods to treat and reuse hydraulic fracturing wastewater are still under development, so injection remains the major disposal method, despite concerns over associations between injection disposal wells and increased seismicity and earthquakes (Ellsworth et al., 2015; Weingarten et al., 2015). More than 95% of produced wastewater in the US is injected for final disposal (US EPA, 2015; Clark and Veil, 2009), though centralized wastewater disposal facilities handle a more significant portion of wastewater in the Marcellus Shale region specifically (US EPA, 2015; Lutz et al., 2013). Spills and/or discharges of wastewater have been shown to increase: 1) fracturing chemical concentrations in local water supplies and sediments (DiGiulio et al., 2011; Rozell and Reaven, 2012; Skalak et al., 2014), 2) heavy metals in drinking water (Fontenot et al., 2013; Jackson et al., 2013; Osborn et al., 2011), and 3) radioactivity, salinity, and total dissolved solids in rivers downstream from treatment plants and/or discharges (Harkness et al., 2015; Hladik et al., 2014; Warner et al., 2013), potentially leading to the production of disinfection byproducts (Harkness et al., 2015; Hladik et al., 2014; Parker et al., 2014). Previous work in our laboratory has reported potential human and animal health concerns via UOG contamination (Kassotis et al., 2014, 2015c; Webb et al., 2014) as well as adverse health outcomes in male C57 mice exposed during gestation to potentially environmentally-relevant concentrations of a hydraulic fracturing chemical mixture (Kassotis et al., 2015b). Because of these health concerns and the many potential contamination pathways (spills during transport to/from sites, improper handling and disposal of wastewater, failure of well casings, etc.), it is important to fill key data gaps in understanding contamination via underground injection activities and potential environmental impacts (US EPA, 2015).

As such, the goals of this study were to characterize the endocrine disrupting activities of water samples collected from a site where the chemical analyses indicated release of UOG wastewater had occurred and to ascertain potential health risks. Due to the high degree of conservation in nuclear receptor pathways (Diamanti-Kandarakis et al., 2009), in vitro screens such as reporter gene assays and yeast receptor screens are commonly used to assess potential health effects in human and wildlife populations (Naylor, 1999; Soto et al., 2006). These in vitro screens can more easily assess potential threats to human and environmental health than more costly and time-consuming animal studies, since the ability of a chemical to interfere with any aspect of hormone action is a clear indicator of potential resultant health outcomes (Zoeller et al., 2012). Mammalian reporter gene assays are often used due to high sensitivity and the translational potential of results (Naylor, 1999; Soto et al., 2006). Yeast receptor screens tend to be less sensitive, though are less susceptible to toxicity (Leusch et al., 2010). Due to these factors, we opted to couple mammalian and yeast bioassays to assess differences between the systems and to ensure that toxicity concerns would not prevent characterization of EDC activities at these sites. We further used authentic standards to convert receptor activities to equivalent concentrations of well-described control chemicals, facilitating the translation of in vitro results, as exposure to EDCs has been linked to a number of negative health outcomes in laboratory animals at environmentally relevant concentrations, wildlife and humans (Akingbemi and Hardy, 2001; Christiansen et al., 2008; Kelce and Wilson, 1997; Kidd et al., 2007; Mendiola et al., 2011; Sumpter and Jobling, 1995; Tyler et al., 1998).

The site examined herein was a West Virginia wastewater injection disposal facility that included an injection disposal well, several lined holding ponds and brine storage tanks, and a small stream that flows through the site (Fig. 1). This stream flows into the Wolf Creek downstream, and eventually into the New River, a drinking water source for local communities and important recreational area. A second tributary of Wolf Creek was identified as a background, non-impacted site, and samples were collected from both streams and assessed for agonist and antagonist activities for the estrogen (ER), androgen (AR), progesterone (PR), glucocorticoid (GR), and thyroid (TR) receptors. From our prior work with individual UOG chemicals and mixtures, we hypothesized that the disposal facility

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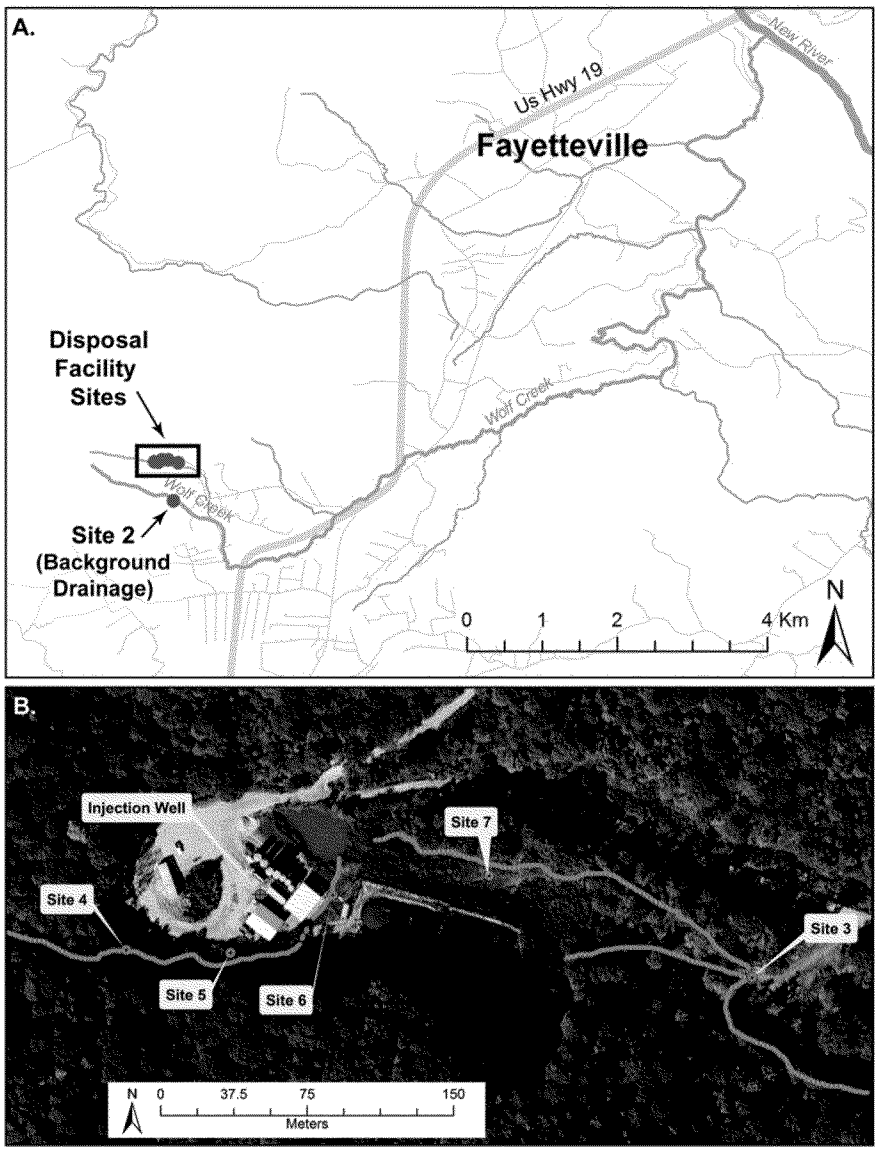

Fig. 1. Map of sampling locations. Map of sampling locations near Fayetteville, WV within the Wolf Creek watershed (A) and specific s te s (B) in a stream running adjacent to a class II disposal facility. Panel A shows that Site 2 was located in a separate drainage from the disposal facility sites (outlined in black box), which are shown in panel B (Sites 4, 5, 6, 7 a n d 3). In panel B, the blue line highlights the stream as it flows through the disposal site. Water samples were not collected at Sites 1 and 5 for the work described herein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Source: Esri. DigitalGlobe, GeoEy, i-cubed, Earthstar Geographies, CNES/Airbus DS, USDA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo, and the GIS User Community.

may contribute antagonist activities to the stream that could impact local health.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2; estrogen agonist, 98% pure), ICI 182,780 (estrogen antagonist, 98% pure), 4,5α-dihydrotestosterone (DHT; androgen agonist, ≥97.5% pure), flutamide (androgen antagonist, 100% pure), 3,3′,5-triiodo-L-thyronine (T3; thyroid agonist, ≥95% pure), progesterone (P4; progesterone agonist, ≥99% pure), mifepristone (glucocorticoid/progesterone antagonists, ≥98% pure), dexamethasone (DXM; glucocorticoid agonist, 99.5% pure), and hydrocortisone (glucocorticoid agonist, 98% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 1-850 (thyroid antagonist, ≥95% pure) was purchased from EMD Millipore (Billerica, MA). Stock solutions were prepared at 10 mM in HPLC-grade methanol and stored at − 20 °C, (except T3 and 1-850, which were prepared in dimethylsulfoxide; DMSO), and then diluted in respective solvents to required working solution concentrations.

2.2. Selection of sample sites and controls

Water samples (n = 6) were collected from surface water sites in June 2014 (Fig. 1, SI 2), including four sites associated with the disposal well: one sample collected upstream from the injection well (Background, Site 4), one near the injection well (Site 6), and another two samples downstream (Sites 7 and 3). Samples were collected from an additional background site in a separate drainage ("background drainage") with no known oil and gas wastewater inputs (Site 2). For additional information about the sampling sites, see Akob et al. (2016, unpublished results).

Process controls were prepared using one liter of Fisher HPLC-grade water (Fisher Scientific catalog # WFSK-4) and followed the same processing and analysis procedures used for all experimental samples. Process controls were included in assays to assess any receptor activities contributed by the solid phase extraction process.

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2.3. Grab sample collection

All samples for Lab One (mammalian assays) were collected in one-liter amber glass bottles (Thermo Scientific catalog # 05-719-91) and samples for Lab Two (yeast assays) were collected in one-liter amber glass bottles (C&G Containers and Scientific Supplies, Lafayette, LA), all certified to meet the EPA standards for metals, pesticides, volatiles, and non-volatiles. Surface water samples were taken from flowing stream water by submerging bottles, filling completely, and capping without headspace. Samples for mammalian assays were preserved in the field by adding 1 g of sodium azide. A duplicate sample was collected at Site 3 and processed separately as an internal control (Supplemental information 2). Field blanks were collected at Site 3, and contained one liter of laboratory control water, opened and briefly exposed to the air, and then preserved and processed in the same manner as field samples. All samples were stored on ice in the field, shipped in coolers overnight to analysis labs, corrected to pH 3 with 6 N HCl (yeast assays only), stored at 4 °C in the respective laboratories, and were processed within two weeks of collection. All analyses were performed blinded to sample identification using non-identifiable coded IDs, and chain of custody procedures followed throughout the shipping and receiving processes.

2.4. Extraction of water samples

The two laboratories followed similar but distinct solid-phase extraction (SPE) protocols, both utilizing Oasis HLB glass cartridges (Waters # 186000683) after a pre-filtration step using glass-fiber filters. Cartridges for mammalian assays were conditioned with 100% HPLC-grade methanol and 100% HPLC-grade H_2O. Water samples (1 L) were loaded onto the cartridge and washed with 5 mL of 5% methanol. Cartridges were then removed from the vacuum manifold and elution was performed with three 1-mL additions of 100% methanol into amber glass vials. A DMSO "keeper" at 50 μL was added to each vial before dry-down under a gentle stream of nitrogen gas and subsequent reconstitution in 200 μL of pure methanol, creating stock concentrations of 4,000× the original water concentration (80% methanol, 20% DMSO). Solid-phase extractions for yeast assay samples were performed as described previously (Ciparis et al., 2012). Briefly, cartridges were conditioned with 100% ethyl acetate, 50:50 methanol: dichloromethane, 100% methanol, and then pH-3 HPLC-grade deionized water. Water samples (800 mL) were loaded onto the cartridge and column dried for at least 30 min following loading. Elution was performed with 6 mL methanol into one glass tube and 6 mL 50:50 methanol:dichloromethane into a second. Samples were dried under nitrogen gas and pooled, and subsequent reconstitution in 1 mL of pure methanol created stock concentrations of 800× the original water concentration.

Reconstituted samples were stored at − 20 °C, protected from light, until tested. In order to be applied to cells, stock samples were diluted 100 and/or 1000-fold in tissue culture medium, creating final concentrations, in contact with mammalian and yeast cells, of 40×/4× the original water concentration for mammalian assays and 8× for yeast assays. Select SPE extracts from yeast assays were also tested in mammalian assays and exhibited equivalent activities to mammalian extracts.

2.5. Mammalian hormone receptor activity assays

Ishikawa cells (Sigma cat # 99040201) were maintained and transiently transfected with plasmids as described previously (Kassotis et al., 2015b; Kassotis et al., 2014) for ER alpha, AR, PR B, GR, and TR beta. Cells were induced with dilution series of the positive/negative controls (SI 3) or of the water sample extracts, diluted in medium using a 1% methanol vehicle. Each treatment concentration for each sample was performed in quadruplicate within each assay and each assay was repeated three times.

Receptor activities were compared to 1% methanol or 0.1% DMSO vehicle controls as necessary, depending on vehicle used. Chemical response was set as a fold induction relative to this vehicle control, prior to calculating relative responses to control agonists and/or antagonists. Agonist activities were then calculated as a percent activity relative to the maximal positive control responses of 200 pM E2, 3 nM DHT, 100 pM P4, 100 nM T3, and 100 nM DEX, for ER, AR, PR, TR, and GR receptor assays, respectively. Antagonist activities were calculated as a percent suppression or enhancement of the positive controls at their EC50s (concentration required to exhibit half of maximal activity): 20 pM E2, 300 pM DHT, 30 pM P4, 2 nM T3, and 5 nM DEX, respectively. Equivalence values were then determined for each sample with significant activity (based on paired t-test) using these percent activities relative to positive control agonist and antagonist dose response curves (Supplemental Fig. 1). Non-significant percent activities, while reported in Fig. 2, did not have equivalent concentrations calculated for Figs. 3 and 4.

2.6. Sample toxicity

The two laboratories followed distinct toxicity test protocols. Mammalian assays assessed toxicity as follows: CMV-β-Gal activity was used in ER assays as a marker of cell number, and also used as a surrogate marker for sample toxicity as described previously (Kassotis et al., 2014). Any sample found to have deviated N15% from the activity of the vehicle and that exhibited a significant difference (based on paired t-test) was deemed toxic and excluded from antagonist analysis. As antagonist assays measure the reduction in luciferase expression, toxicity cannot be unpaired from antagonist action. As such, any sample found to exhibit toxicity at the 40× concentration (Sites 3 and 7) were excluded and only tested for antagonism at 4× where no significant toxicity was observed for any sample. Yeast assays assessed toxicity at 8× water concentration using yeast strain BLYR (Sanseverino et al., 2009). Strain BLYR was grown to an OD_{600} of 0.5 and was then added to samples and incubated for 4 h at 30 °C. Toxicity was expressed as the percent reduction in bioluminescence relative to vehicle control (2.5% methanol). Samples were considered toxic if a 10% reduction (or greater) in bioluminescence was observed. No toxicity was observed for any samples in the yeast system.

2.7. Yeast bioreporter assays

The bioluminescent yeast estrogen screen (BLYES) was used to quantitatively assess ER alpha activity relative to 17β-estradiol. Strain BLYES was purchased from 490 BioTech. Yeast strains DSY-1555 and MCY-105 were used to assess AR and GR, respectively. These yeast reporter strains were obtained from Marc Cox (University of Texas at El Paso). Detection limits for these yeast strains in the culture conditions described below are BLYES, 0.31 ng/L of 17β-estradiol; DSY-1555, 0.80 ng/L of dihydroxytestosterone; DSY-105, 0.05 ng/L of hydrocortisone.

The BLYES assay was performed as described previously (Balsiger et al., 2010; Ciparis et al., 2012) with some modifications. Strains DSY-1555 and MCY-105 were grown in synthetic complete media lacking lysine, uracil and tryptophan (SC-LUW) or uracil, tryptophan and histadine (SC-UWH), respectively. Yeast was grown at 30 °C in a rotary incubator for 48 h. Yeast was diluted to an OD_{600} of 0.25 and 95 μL was added to wells of solid bottom white microplates (Costar). Standards (1.5×10^4–8 ng/well) and samples (5 μL) were then added and plates were incubated at 30 °C for 4 h. After this incubation, 100 μL of Tropix GalScreen in Buffer B (Applied Biosystems, Foster City, CA) was added to all wells and the plate incubated for an additional 2 h at 28 °C. The hormone induced chemiluminescent signal

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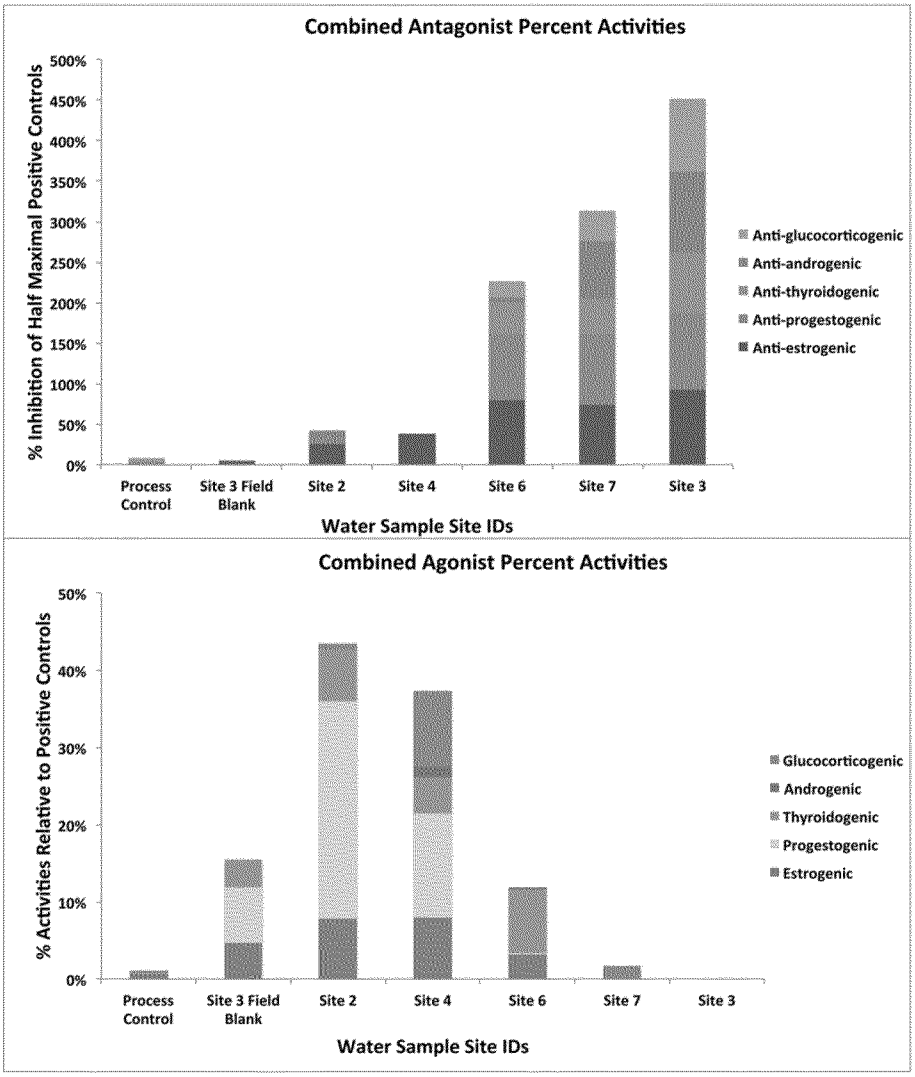

Fig. 2. Agonist and antagonist combined receptor activities of 4× surface water samples associated with injection well site via mammalian reporter gene assay. Combined total receptor activities for each water sample at 4× concentration. Combined total antagonist activities (A) as percent suppression of half maximal positive control response for each receptor. Combined total agonist receptor activities (B) as percent activity relative to maximal positive control response for each receptor. Results from duplicate samples, collected at Sites 7 and 3, were averaged and presented as one value for these sites. Samples are in order of degree of potential impact from the disposal facility: Process control, Site 3 field blank, Site 2 background stream, Site 4 upstream background, Site 6 adjacent to the injection well, Site 7 near former impoundment ponds, and Site 3 downstream of facility.

was then measured on a SpectraMax M4 microplate reader (Molecular Devices) in luminescence mode (1000 ms integration time).

3. Results

3.1. Antagonist receptor activities of water extracts

Increasing and near maximal antagonist activities were noted on and downstream of the disposal facility (Fig. 2). Site 6 (adjacent to the injection well) exhibited near maximal (N80%) antagonism for ER and PR, with antagonism for AR, GR, and TR increasing in Site 7 (adjacent to the impoundment ponds) and further in Site 3 (downstream of site; Fig. 2A). The background samples from the reference stream (Site 2) and from upstream of the disposal facility (Site 4) exhibited non-significant antagonism. Equivalence values were calculated based on positive control antagonists. Anti-AR and anti-TR equivalent activities were the highest measured, with levels reaching 700 μg EQ/L for each (flutamide and 1–850 equivalences, respectively; Fig. 3B, E). Anti-PR activity reached 5.5 μg mifepristone-EQ/L at Site 3 (Fig. 3C), anti-GR reached approximately 600 ng mifepristone-EQ/L (Fig. 3D), and anti-ER reached approximately 200 ng ICI-EQ/L (Fig. 3A).

3.2. Agonist receptor activities of water extracts

Low levels of background agonist activities (approximately 10-times lower than antagonist activities) were observed in the reference stream, Site 2, and the sample collected upstream from the wastewater disposal facility, Site 4 (Figs. 2B, 4). The main agonist activity was for PR, at 28% and 13% activity relative to the positive control at Sites 2 and 4, respectively (Fig. 2B). The field blank collected at Site 3 exhibited some non-significant agonist activities, and agonist activities were largely or completely gone in the three samples collected on or downstream from the injection disposal facility (Sites 6, 7, 3). Equivalence values at Sites 2, 4, and occasionally the field blank and background Site 6 exhibited low levels of agonist activities (Fig. 4). TR equivalence was highest, with levels as high as 5 ng T3-equivalents per liter water (T3-EQ/L; Fig. 4E), while both AR and GR equivalences were approximately 1 ng EQ/L (Fig. 4B, D). PR equivalence was between 115 and 150 pg P4-EQ/L (Fig. 4C), while ER was the lowest detected activity at 15–18 pg E2-EQ/L (Fig. 4A). All sites potentially impacted by injection fluids from the disposal well operations (Sites 6, 7, and 3) exhibited less agonist activities and considerably more antagonist activities than background samples.

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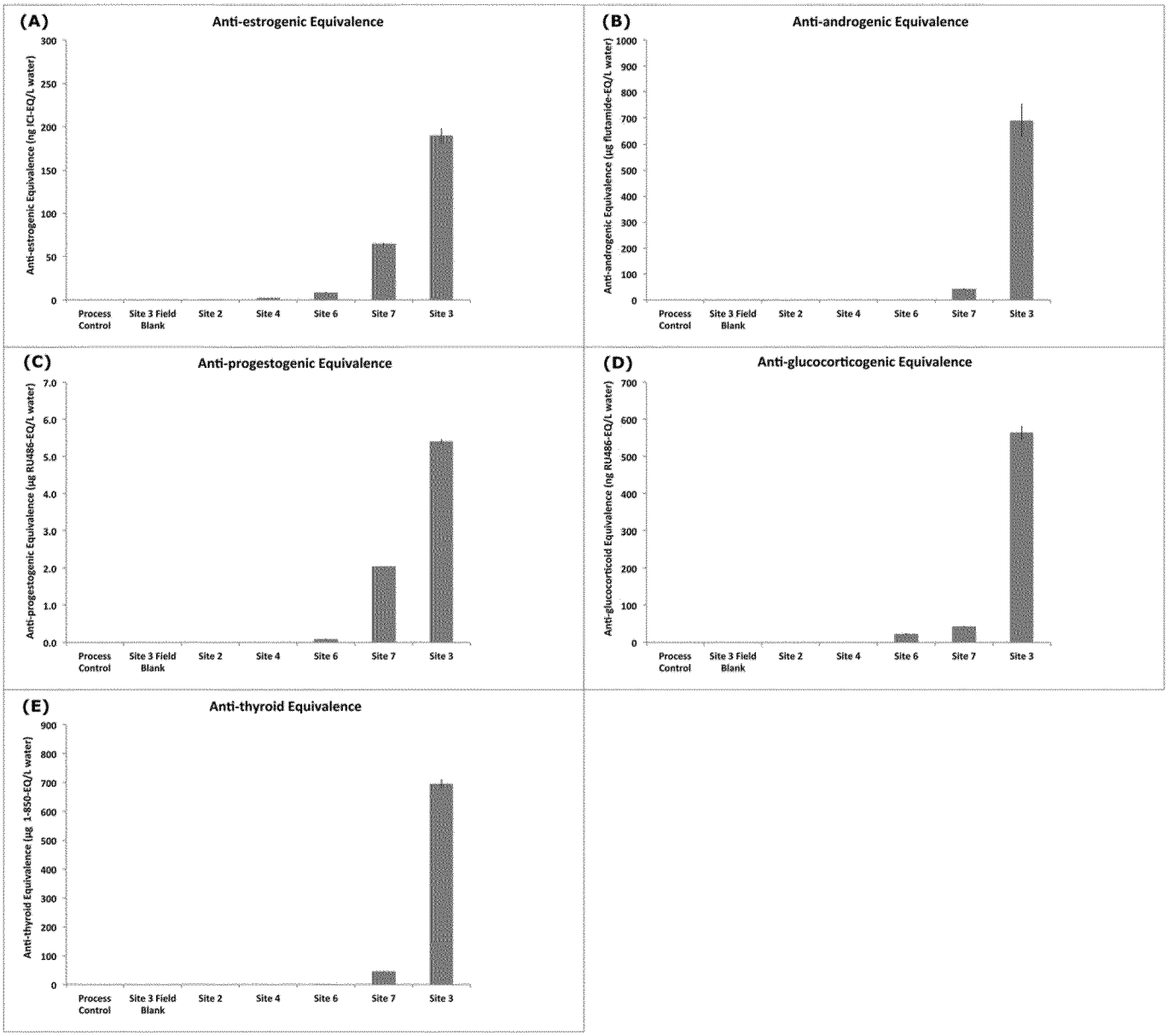

Fig. 3. Antagonist equivalence values of surface water samples associated with injection well site via mammalian reporter gene assay. Antagonist equivalences ± SEM calculated as an equivalent positive control antagonist concentration for: (A) anti-estrogenic (ICI), (B) anti-androgenic (flutamide), (C) anti-progestogenic (mifepristone), (D) anti-glucocorticoid (mifepristone), and (E) anti-thyroid (1–850) at each sample site at 4× concentration. Results from duplicate samples, collected at Sites 7 and 3, were averaged and presented as one value for these sites. Equivalence values calculated only for samples exhibiting significant activity as described in the methods. Samples in order of increasing potential impact from facility.

ER, AR, and GR activities were also assessed in yeast reporter gene assays (SI 1). No significant GR activity was measured at any site. ER activity was only observed at the downstream Sites 7 and 3, with equivalent activities of approximately 350 and 650 pg E2-EQ/L (SI 1A). AR activity was likewise only measured at Sites 7 and 3, with equivalent concentrations between 1.3 and 1.5 ng DHT-EQ/L (SI 1B). No ER or AR agonist activities were observed at these sites in the mammalian reporter gene assays, though they exhibited the highest antagonism (Fig. 3).

3.3. Toxicity assessment of water extracts

Samples were assessed for toxicity via CMV-β-Gal activity in the ER activity screen in Ishikawa human cells, as described and validated previously (Kassotis et al., 2014). Briefly, a constitutively active promoter, CMV-β-Gal, was transfected into all cells alongside the reporter and receptor constructs for each experimental system. Any cell thus transfected with this promoter produced beta galactosidase, and this could be measured as a marker for cell number and thus also as a surrogate marker for cell toxicity (Kassotis et al., 2014). Sites 7 and 3 both exhibited moderately high toxicity (N60% inhibition of beta-galactosidase production) at the 40× test concentration (Fig. 5). None of the 4× concentrations of these samples exhibited significant toxicity. Agonist and antagonist activities were only reported for samples without significant toxicity, so 4× water concentration values were used to report all activities. No toxicity was observed for any samples at 8× water concentration in the yeast reporter gene assay.

4. Discussion

We measured significantly greater EDC activity on and downstream of the disposal facility (Sites 6, 7, and 3) relative to reference sites (Sites 2 and 4). The impacted sites exhibited considerably greater antagonist activities than background samples (Figs. 2, 3, 4). These samples included Site 6 collected directly adjacent to the injection well, Site 7 collected

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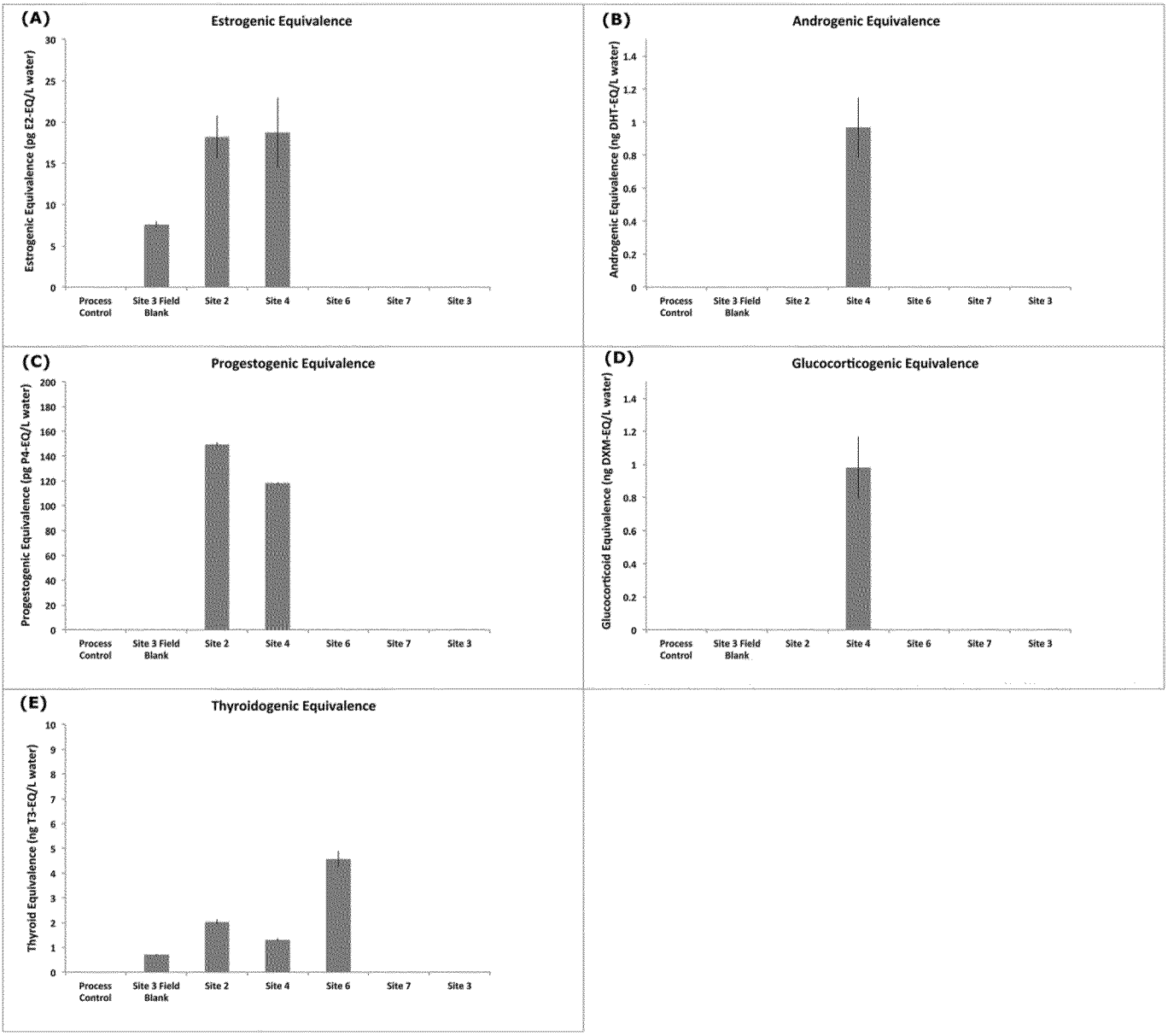

Fig. 4. Agonist equivalence values and receptor activities of surface water samples associated with injection well site via mammalian reporter gene assay. Agonist equivalences ± SEM calculated as an equivalent positive control agonist concentration for: (A) estrogenic (E2), (B) androgenic (DHT), (C) progestogenic (progesterone), (D) glucocorticoid (dexamethasone), and (E) thyroidogenic (T3) at each sample collection site at 4× concentration. Results from duplicate samples, collected at Sites 7 and 3, were averaged and presented as one value for these sites. Equivalence values calculated only for samples exhibiting significant activity as described in the methods. Samples in order of increasing potential impact from facility.

immediately next to the former wastewater containment ponds, and Site 3 collected downstream from the entire disposal facility. Site 3 exhibited the most antagonism, likely due to this sample receiving drainage from the entire facility. The most impacted samples, Sites 7 and 3, also exhibited toxicity in the mammalian cell culture system at the 40× concentration, but not at the 4× concentration used to measure EDC bioactivity, nor at 8× in the yeast cell culture system.

As a class II injection well, this site is permitted to accept wastewater from unconventional oil and natural gas extraction. However, this site may accept wastewater and fluids from other industries as well, and the hormonal activity profile exhibited may be due in part to other sources. As such, caution should be taken in the extrapolation of these results to unconventional oil and gas activities specifically. To address this concern, research performed concurrently (Akob et al., 2016 and Orem et al., 2016, unpublished results) describes in detail the analytical and geochemical profiling that identified inorganic and organic constituents indicative of UOG wastewater at these sites. Specifically, elevated conductivity, sodium, chloride, and barium concentrations, and strontium isotopes suggest that the contamination profile is specifically due to the handling of UOG wastewater from shale gas and coal bed methane production (SI 2, Akob et al., 2016, unpublished results). In addition, numerous organic chemicals were identified in water and sediments downstream of the injection facility, many associated with UOG operations (Orem et al. 2016, unpublished results).

Several known hormonally active compounds were detected in the water near the injection disposal facility. However, differences in sensitivity and efficacy between assay systems prevent clear associations with degree of effects. Detected at Sites 3 and 7, tris(1-chloro-2-propyl)phosphate has been reported to act as an antagonist for the androgen (Weiss et al., 2011) and thyroid (Farhat et al., 2013) receptors. Detected at Site 3, 2-(2-butoxyethoxy)-ethanol (diethylene glycol methyl ether) has been tested by our lab previously and exhibited antagonistic activities for ER, AR, and GR (Kassotis et al., 2015b), though only at concentrations approximately 100-times above the 0.54 μg/L

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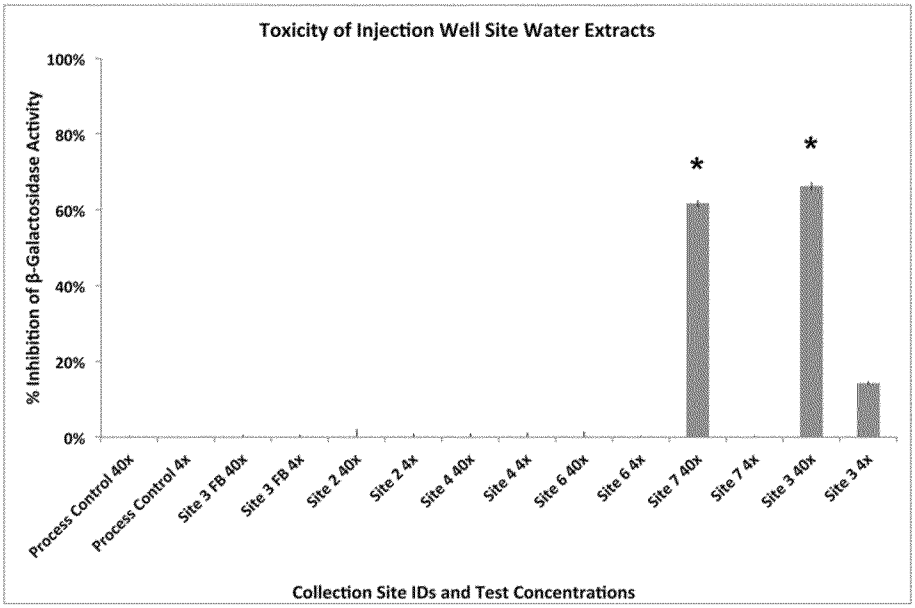

Fig. 5. In vitro toxicity of injection well site surface water samples. Toxicity as per inhibition of constitutively active promoter, CMV-β-Gal, measured as percent inhibition of β-galactosidase by 40× and 4× concentrations of each water sample ± SEM in Ishikawa cells. Significant inhibition designated by N15% inhibition of signal and significantly lower response than vehicle control, as per t-test. Results from duplicate samples, collected at Sites 7 and 3, were averaged and presented as one value for these sites. *p b 0.05 and N15% inhibition of vehicle control.

in the water at this site. Notably, many EDCs are hydrophobic and partition more readily into sediments, resulting in lower concentrations in water samples (Lai et al., 2000; Langston et al., 2005; Petrovic et al., 2001; Pojana et al., 2007). As such, sediments at Sites 3 and 7 contained 16 and 65 of the chemicals assessed, respectively. Future work should fractionate water samples to gain a clearer understanding of the chemicals driving the majority of antagonist activities.

The bioactivities we measured in water sample extracts from impacted sites were within the range known to impact the health of aquatic organisms. Specifically, anti-PR, anti-GR, anti-AR, anti-TR, and anti-ER activities were approximately 1000, 100, 30, 15, and 6 times greater than those known to disrupt the endocrine system in aquatic organisms, respectively (Bhatia et al., 2015; Bluthgen et al., 2013a, 2013b; Navarrete-Ramirez et al., 2014; Roepke et al., 2005). For example, 30 ng/L ICI inhibits development of sea urchins (Roepke et al., 2005), 25 μg/L flutamide can induce vitellogenin production in fish (Bhatia et al., 2015), 5 ng/L mifepristone impacts egg production, disrupts folliculogenesis, and alters gene expression in zebrafish (Bluthgen et al., 2013a, 2013b), and 46 μg/L 1–850 can alter gene expression in tilapia (Navarrete-Ramirez et al., 2014). Notably, antagonist equivalent activities reported downstream of the disposal facility (Sites 7 and 3) were above levels associated with adverse health effects in aquatic organisms for all five receptors. In many cases, even with considerable dilution, levels of endocrine disrupting contaminants would still be capable of disrupting the development of fish, amphibians, and other aquatic organisms.

Impacted sites largely contained minimal agonist activity, and generally occurred below levels known to impact aquatic wildlife. Agonist activities in water sample extracts from reference sites were also below those known to cause adverse health effects in aquatic organisms, to the best of our knowledge. Importantly, despite this low potential for disruption through single receptor mechanisms, adverse health effects may result from disruption of several receptor pathways simultaneously. For example, Runnalls et al. recently reported that ER, AR, and PR agonist pathways could all result in inhibition of egg production in fathead minnows through separate mechanisms (Runnalls et al., 2015), suggesting that some endpoints may require a more comprehensive approach than assessing equivalent concentrations for individual receptors.

Our lab has previously reported anti-ER and anti-AR equivalences in surface and groundwater collected from drilling-dense sites with a history of hydraulic fracturing fluid spills in Colorado (Kassotis et al., 2014) and surface water impacted by wastewater effluent in Missouri (Kassotis et al., 2015a), though equivalent ER and AR agonist and antagonist concentrations were much lower than those described herein. For example, wastewater effluent impacted streams in Missouri exhibited up to 19 ng/L ICI equivalence (anti-ER) and 48 μg/L flutamide equivalence (anti-AR), approximately 10 and 14-fold lower activities than detected in this study. Other researchers have utilized similar in vitro screens to assess the EDC activities contributed by various anthropogenic sources to water, though varying positive controls and assay sensitivities complicate comparisons. However, wastewater is a well-described source of anti-androgens, with raw sewage containing up to mg/L levels of flutamide equivalence (Ma et al., 2013). Researchers assessing the Lambro River in Italy, heavily contaminated with domestic and industrial wastewater and agricultural run-off, reported 370–4700 μg/L flutamide equivalences (Urbatzka et al., 2007). Similarly, assessment of the Pearl River System in China, heavily contaminated by effluent and raw sewage from four major wastewater treatment plants, exhibited 20–935 μg/L flutamide equivalence and up to 1.3 mg/L tamoxifen equivalence (anti-ER; Zhao et al., 2011). While tamoxifen exhibits agonist activity in our uterine bioassay system and thus cannot be readily compared, the anti-androgenic activities were similar to ours.

Differences noted between the activities exhibited in the mammalian and yeast screens are likely due to several known factors. Many of the tissue-specific effects of EDCs in mammalian systems are due to the varied expression of coregulatory proteins recruited by the ligand-bound receptor complex (Diel, 2002; Shang and Brown, 2002). Yeast receptor screens lack many of these coregulators, and chemicals that act as antagonists in mammalian systems can act as agonists in a yeast system (Lyttle et al., 1992; Sohoni and Sumpter, 1998; Urbatzka et al., 2007). Yeast cells also lack some enzymes that are commonly expressed in mammalian cells, preventing bioactivation of some chemicals routinely observed in mammalian cells (Bovee et al., 2007). Lastly, the permeability of chemicals through the cell wall in yeast is different from mammalian cell membranes, resulting in differential sensitivity to various chemicals (Wilson et al., 2004).

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5. Conclusions

In conclusion, we report high levels of EDC activities in surface water extracts associated with a wastewater injection disposal facility. The most impacted sites were on and downstream from the disposal facility (Sites 6, 7, and 3), and exhibited considerably more antagonist activities and less agonist activities than background samples (Figs. 2, 3, 4). The most impacted samples, Sites 7 and 3, also exhibited toxicity in the mammalian cell culture system at the 40× concentration, but not at the 4× concentration that we used to measure EDC activity. Importantly, the water leaving this site exhibited nuclear receptor equivalent activities that are known to result in adverse health effects in aquatic organisms and other animals. While Wolf Creek flows into the New River, a drinking water resource, this sampling occurred approximately 5 miles upstream from the confluence. Further work should assess how the magnitude of EDC effects changes with distance from the site in order to better assess potential human and animal health threats from exposure. Given the large number (N140.000) of class II injection wells currently operating in the United States, this should be viewed as a case study of environmental impacts that may be evident at other injection disposal facilities as well. Further work, including higher tier receptor disruption screens (whole cell activity as well as fish, amphibian, and mammalian whole animal assessments) should be applied to confirm these results and assess water quality surrounding these facilities in a more comprehensive manner.

Competing financial interest declaration

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.03.113

References

Akingbemi, B.T., Hardy, M.P., 2001. Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. Ann. Med. 33, 13.

Akob, D.M., Cozzarelli, I.M., Dunlap, D.S., Rowan, E.L., Lorah, M.M., 2015. Organic and inorganic composition and microbiology of produced waters from Pennsylvania shale gas wells. Appl. Geochem. 60, 116–125.

Balsiger, H.A., de la Torre, R., Lee, W.Y., Cox, M.B., 2010. A four-hour yeast bioassay for the direct measure of estrogenic activity in wastewater without sample extraction, concentration, or sterilization. Sci. Total Environ. 408, 1422–1429.

Bhatia, H., Kumar, A., Du, J., Chapman, J.C., McLaughlin, M.J., 2015. Co-treatment with the non-steroidal anti-androgen drug, flutamide and the natural estrogen, 17beta-estradiol does not lead to additive reproductive impairment in juvenile Murray rainbowfish (Melanotaenia fluviatilis). J. Appl. Toxicol. 35, 1241–1253.

Bluthgen, N., Castiglioni, S., Sumpter, J.P., Fent, K., 2013a. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (Danio rerio): 1. Reproductive and early developmental effects. Aquat. Toxicol. 144-145, 83–95.

Bluthgen, N., Sumpter, J.P., Odermatt, A., Fent, K., 2013b. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (Danio rerio): 2. Gene expression analysis and in vitro activity. Aquat. Toxicol. 144-145, 96–104.

Bolden, A.L., Kwiatkowski, C.F., Colborn, T., 2015. New look at BTEX: are ambient levels a problem? Environ. Sci. Technol. 49, 5261–5276.

Bovee, T.F.H., Helsdingen, R.J.R., Hamers, A.R.M., Van Duursen, M.B.M., Nielen, M.W.F., Hoogenboom, R.L.A.P., 2007. A new highly specific and robust yeast androgen bioassay for the detection of agonists and antagonists. Anal. Bioanal. Chem. 389, 1549–1558.

Chen, J., Ahn, K.C., Gee, N.A., Ahmed, M.I., Duleba, A.J., Zhao, L., et al., 2007. Triclocarban enhances testosterone action: a new type of endocrine disruptor? Endocrinology 149, 1173–1179.

Christiansen, S., Scholze, M., Axelstad, M., Boberg, J., Kortenkamp, A., Hass, U., 2008. Combined exposure to anti-androgens causes markedly increased frequencies of hypospadias in the rat. Int. J. Androl. 31, 241–248.

Ciparis, S., Iwanowicz, L.R., Voshell, J.R., 2012. Effects of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams. Sci. Total Environ. 414, 268–276.

Clark, C., Veil, J., 2009. Produced water volumes and management practices in the United States. Argonne National Laboratory (ANL). Available at: http://www.ipd.anl.gov/anlpubs/2009/07/64622.pdf (accessed 26 January 2016).

Colborn, T., Kwiatkowski, C., Schultz, K., Bachran, M., 2011. Natural gas operations from a public health perspective. Hum. Ecol. Risk. Assess. 17, 1039–1056.

Deutch, J., Holditch, S., Krupp, F., McGinty, K., Tierney, S., Yergin, D., et al., 2011. The Secretary of the Energy Board Shale Gas Production Subcommittee Ninety-Day Report. Available at: http://www.shalegas.energy.gov/resources/081811_90_day_report_final.pdf (accessed 26 January 2016).

Diamanti-Kandarakis E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., et al., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342.

Diel, P., 2002. Tissue-specific estrogenic response and molecular mechanisms. Toxicol. Lett. 127, 217–224.

DiGiulio, D.C., Wilkin, R.T., Miller, C., Oberley, G., 2011. Investigation of Ground Water Contamination Near Pavillion. Environmental Protection Agency, Wyoming, Draft Report. Available: http://www.epa.gov/sites/production/files/documents/EPA_ReportOnPavillion_Dec-8-2011.pdf (accessed: 26 January 2016).

Ellsworth, W.L., Llenos, A.L., McGarr, A.F., Michael, A.J., Rubinstein, J.L., Mueller, C.S., et al., 2015. Increasing seismicity in the U.S. midcontinent: implications for earthquake hazard. Science 34, 618–626.

Engle, M., Cozzarelli, I.M., Smith, B.D., 2014. USGS investigations of water produced during hydrocarbon reservoir development. U.S. Geological Survey Fact Sheet 2014-3104 Availableat: http://pubs.usgs.gov/fs/2014/3104/pdf/fs2014-3104.pdf (accessed 26 January 2016).

Farhat, A., Crump, D., Chiu, S., Williams, K.L., Letcher, R.J., Gauthier, L.T., et al., 2013. In ovo effects of two organophosphate flame retardants–TCPP and TDCPP–on pipping success, development, mRNA expression, and thyroid hormone levels in chicken embryos. Toxicol. Sci. 134, 92–102.

Fontenot, B.E., Hunt, L.R., Hildenbrand, Z.L., Carlton Jr., D.D., Oka, H., Walton, J.L., et al., 2013. An evaluation of water quality in private drinking water wells near natural gas extraction sites in the Barnett Shale formation. Environ. Sci. Technol. 47, 10032–10040.

Harkness, J.S., Dwyer, G.S., Warner, N.R., Parker, K.M., Mitch, W.A., Vengosh, A., 2015. Iodide, bromide, and ammonium in hydraulic fracturing and oil and gas wastewaters: environmental implications. Environ. Sci. Technol. 49, 1955–1963.

Hayes, T.B., 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Natl. Acad. Sci. 99, 5476–5480.

Hladik, M.L., Focazio, M.J., Engle, M., 2014. Discharges of produced waters from oil and gas extraction via wastewater treatment plants are sources of disinfection by-products to receiving streams. Sci. Total Environ. 466-467, 1085–1093.

Jackson, R.B., Vengosh, A., Darrah, T.H., Warner, N.R., Down, A., Poreda, R.J., et al., 2013. Increased stray gas abundance in a subset of drinking water wells near Marcellus shale gas extraction. Proc. Natl. Acad. Sci. 110, 11250–11255.

Jansen, M.S., Nagel, S.C., Miranda, P.J., Lobenhofer, E.K., Afshari, C.A., McDonnell, D.P., 2004. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. Proc. Natl. Acad. Sci. 101, 7199–7204.

Kassotis, C.D., Alvarez, D.A., Taylor, J.A., vom Saal, F.S., Nagel, S.C., Tillitt, D.E., 2015a. Characterization of Missouri surface waters near point sources of pollution reveals potential novel atmospheric route of exposure for bisphenol A and wastewater hormonal activity pattern. Sci. Total Environ. 524-525, 384–393.

Kassotis, C.D., Klemp, K.C., Vu, D.C., Lin, C.H., Meng, C.X., Besch-Williford, C.L., et al., 2015b. Endocrine-disrupting activity of hydraulic fracturing chemicals and adverse health outcomes after prenatal exposure in male mice. Endocrinology 156, 4458–4473.

Kassotis, C.D., Tillitt, D.E., Davis, J.W., Hormann, A.M., Nagel, S.C., 2014. Estrogen and androgen receptor activities of hydraulic fracturing chemicals and surface and ground water in a drilling-dense region. Endocrinology 155, 897–907.

Kassotis, C.D., Tillitt, D.E., Lin, C.H., McElroy, J.A., Nagel, S.C., 2015c. Endocrine-disrupting chemicals and oil and natural gas operations: potential environmental contamination and recommendations to assess complex environmental mixtures. Environ. Health Perspect. 124, 256–264.

Please cite this article as: Kassotis, C.D., et al., Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site, Sci Total Environ (2016), http://dx.doi.org/10.1016/j.scitotenv.2016.03.113

Kelce, W.R., Wilson, E.M., 1997. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. J. Mol. Med. 75, 198–207.
Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., et al., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901.
Lai, K.M., Johnson, K.L., Scrimshaw, M.D., Lester, J.N., 2000. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Environ. Sci. Technol. 34, 3890–3894.
Langston, W.J., Burt, G.R., Chesman, B.S., Vane, C.H., 2005. Partitioning, bioavailability and effects of oestrogens and xeno-oestrogens in the aquatic environment. J. Mar. Biol. Assoc. U. K. 85, 1–31.
Lee, D.S., Herman, J.D., Elsworth, D., Kim, H.T., Lee, H.S., 2011. A critical evaluation of unconventional gas recovery from the Marcellus Shale, Northeastern United States. KSCE J. Civ. Eng. 15, 679–687.
Lester, Y., Ferrer, I., Thurman, E.M., Sitterley, K.A., Korak, J.A., Aiken, G., et al., 2015. Characterization of hydraulic fracturing flowback water in Colorado: implications for water treatment. Sci. Total Environ. 512-513C, 637–644.
Leusch, F.D., de Jager, C., Levi, Y., Lim, R., Puijker, L., Sacher, F., et al., 2010. Comparison of five in vitro bioassays to measure estrogenic activity in environmental waters. Environ. Sci. Technol. 44, 3853–3860.
Lutz, B.D., Lewis, A.N., Doyle, M.W., 2013. Generation, transport, and disposal of wastewater associated with Marcellus Shale gas development. Water Resour. Res. 49, 647–656.
Lyttle, C.R., Damian-Matsumura, P., Juul, H., Butt, T.R., 1992. Human estrogen receptor regulation in a yeast model system and studies on receptor agonists and antagonists. J. Steroid Biochem. Mol. Biol. 42, 677–685.
Ma, D., Chen, L., Lui, R., 2013. Decrease of antiandrogenic activity in gray water and domestic wastewater treated by the MBR process. Environ. Sci. Process Impacts 15, 668–676.
Mendiola, J., Stahlhut, R.W., Jørgensen, N., Liu, F., Swan, S.H., 2011. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. Environ. Health Perspect. 119, 958–963.
Navarrete-Ramirez, P., Luna, M., Valverde, R.C., Orozco, A., 2014. 3,5-di-iodothyronine stimulates tilapia growth through an alternate isoform of thyroid hormone receptor beta1. J. Mol. Endocrinol. 52, 1–9.
Naylor, L.H., 1999. Reporter gene technology: the future looks bright. Biochem. Pharmacol. 58, 749–757.
Osborn, S.G., Vengosh, A., Warner, N.R., Jackson, R.B., 2011. Methane contamination of drinking water accompanying gas-well drilling and hydraulic fracturing. Proc. Natl. Acad. Sci. 108, 5.
Parker, K.M., Zeng, T., Harkness, J., Vengosh, A., Mitch, W.A., 2014. Enhanced formation of disinfection byproducts in shale gas wastewater-impacted drinking water supplies. Environ. Sci. Technol. 48, 11161–11169.
Petrovic, M., Eljarrat, E., Loèpez de Alda, M.J., Barcelo, D., 2001. Analysis and environmental levels of endocrine disrupting compounds in freshwater sediments. Trends Anal. Chem. 20, 637–648.
Pojana, G., Gomiero, A., Jonkers, N., Marcomini, A., 2007. Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. Environ. Int. 33, 929–936.
Roepke, T.A., Snyder, M.J., Cherr, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. Aquat. Toxicol. 71, 155–173.
Rowan, E.L., Engle, M.A., Kraemer, T.F., Schroeder, K.T., Hammack, R.W., Doughten, M.W., 2015. Geochemical and isotopic evolution of water produced from middle Devonian Marcellus Shale gas wells, Appalachian Basin, Pennsylvania. Am. Assoc. Pet. Geol. Bull. 99, 181–206.
Rozell, D.J., Reaven, S.J., 2012. Water pollution risk associated with natural gas extraction from the Marcellus Shale. Risk Anal. 32, 1382–1393.
Runnalls, T.J., Beresford, N., Kugathas, S., Margiotta-Casaluci, L., Scholze, M., Scott, A.P., et al., 2015. From single chemicals to mixtures - reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow. Aquat. Toxicol. 169, 152–167.
Sanseverino, J., Eldridge, M.L., Layton, A.C., Easter, J.P., Yarbrough, J., Schultz, T.W., Sayler, G.S., 2009. Screening of potentially hormonally active chemicals using bioluminescent yeast bioreporters. Toxicol. Sci 107 (1), 122–134.
Shang, Y., Brown, M., 2002. Molecular determinants for the tissue specificity of SERMs. Science 295, 2465–2468.
Skalak, K.J., Engle, M.A., Rowan, E.L., Jolly, G.D., Conko, K.M., AJ, Benthem, et al., 2014. Surface disposal of produced waters in Western and Southwestern Pennsylvania: potential for accumulation of alkali-earth elements in sediments. Int. J. Coal Geol. 126, 162–170.
Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. J. Endocrinol. 158, 327–339.
Soto, A.M., Maffini, M.V., Schaeberle, C.M., Sonnenschein, C., 2006. Strengths and weaknesses of in vitro assays for estrogenic and androgenic activity. Best Pract. Res. Clin. Endocrinol. Metab. 20, 15–33.
Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, 173–178.
The Endocrine Disruption Exchange (TEDX), 2013. TEDX list of potential endocrine disruptors. Available: http://endocrinedisruption.org/endocrine-disruption/tedx-list-of-potential-endocrine-disruptors/overview (accessed 26 January 2016).
Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28, 319–361.
United States Environmental Protection Agency (USEPA), 2015. Assessment of the potential impacts of hydraulic fracturing for oil and gas on drinking water resources. External Review Draft Available: http://ofmpub.epa.gov/eims/eimscomm.get file?p_download_id = 523539 (accessed: 26 January 2016).
Urbatzka, R., van Cauwenberge, A., Maggioni, S., Vigano, L., Mandich, A., Benfenati, E., et al., 2007. Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. Chemosphere 67, 1080–1087.
Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.H., et al., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.
Warner, N.R., Christie, C.A., Jackson, R.B., Vengosh, A., 2013. Impacts of shale gas wastewater disposal on water quality in western Pennsylvania. Environ. Sci. Technol. 47, 11849–11857.
Waxman, H.A., Markey, E.J., DeGette, D., 2011. Chemicals used in hydraulic fracturing. The Committee on Energy and Commerce. House of Representatives, U.S. Available at: http://www.conservation.ca.gov/dog/general_information/Documents/Hydraulic%20Fracturing%20Report%204%2018%2011.pdf (accessed 26 January 2016).
Webb, E., Bushkin-Bedient, S., Cheng, A., Kassotis, C.D., Balise, V., Nagel, S.C., 2014. Developmental and reproductive effects of chemicals associated with unconventional oil and natural gas operations. Rev. Environ. Health 29, 307–318.
Weingarten, M., Ge, S., Godt, J.W., Bekins, B.A., JL, Rubinstein, 2015. High-rate injection is associated with the increase in U.S. mid-continent seismicity. Science 348, 1336–1340.
Weiss, J.M., Simon, E., Stroomberg, G.J., de Boer, R., de Boer, J., Van der Linden, S.C., et al., 2011. Identification strategy for unknown pollutants using high-resolution mass spectrometry: androgen-disrupting compounds identified through effect-directed analysis. Anal. Bioanal. Chem. 400, 3141–3149.
Welshons, W.V., Thayer, K.A., Judy, B.M., Taylor, J.A., Curran, E.M., FS, vom Saal, 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ. Health Perspect. 111, 994–1006.
Wilson, V.S., Bobseine, K., Gray Jr., L.E., 2004. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicol. Sci. 81, 69–77.
Wiseman, H.J., 2008. Untested waters: the rise of hydraulic fracturing in oil and gas production and the need to revisit regulation. Fordham Environ. Law Rev. 20, 115–169.
Yang, M., Park, M.S., Lee, H.S., 2006. Endocrine disrupting chemicals: human exposure and health risks. J. Environ. Sci. Health C. Environ. Carcinog. Ecotoxicol. Rev. 24, 183–224.
Zhao, J.-L., Ying, G.-G., Yang, B., Liu, S., Zhou, L.-J., Chen, Z.-F., et al., 2011. Screening of multiple hormonal activities in surface water and sediment from the Pearl River system, South China, using effect-directed in vitro bioassays. Environ. Toxicol. Chem. 30, 2208–2215.
Zoeller, R.T., Brown, T.R., Doan, L.L., Gore, A.C., Skakkebaek, N.E., Soto, A.M., et al., 2012. Endocrine-disrupting chemicals and public health protection: a statement of principles from the Endocrine Society. Endocrinology 153, 4097–4110.

Please cite this article as: Kassotis, C.D., et al., Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site, Sci Total Environ (2016), http://dx.doi.org/10.1016/j.scitotenv.2016.03.113